

## Prognostic value of tumor-infiltrating lymphocytes in melanoma: a systematic review and meta-analysis

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### ABSTRACT

Tumor-infiltrating lymphocytes (TILs) are associated with prognosis in various tumors. However, it remains controversial whether the presence of TILs is related to an improved prognosis in melanoma. This meta-analysis confirmed the favorable prognostic role of the CD3+, CD4+, CD8+, FOXP3+, and CD20+ TILs in the overall survival of melanoma patients and found an association between the TILs present and improved overall survival. Additionally, subgroup analysis demonstrated that brisk TILs were obviously associated with OS, RFS and DSS/MSS. Thus, TILs may be a predictive biomarker in melanoma. This analysis will provide more insight into the study of TILs and predictive biomarker.

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### Introduction

Despite the advances in global healthcare, malignant tumors remain a major threat to human health. Malignant melanoma is the most aggressive form of skin cancer with unpredictable behavior. Although multiple approaches have been used to treat melanoma, the mortality of melanoma patients has barely improved over the last decades.<sup>1-3</sup> Thus, in addition to Breslow thickness, Clark level, Sentinel lymph node biopsy and serum LDH level, early prediction of the patient prognosis using biomarkers is also a powerful weapon against melanoma.

Melanoma is considered a highly immunogenic tumor, responsive to immunologic manipulation, and the role of the immune response in melanoma has gained much attention in recent years.<sup>4,5</sup> To identify the accurately predictive biomarkers, much focus has been on the biologic properties of cancer cells and immune response in their microenvironment.<sup>6</sup> Tumor-infiltrating lymphocytes (TILs) are a local histopathological reflection of the host's immune response against cancer cells. Currently, TILs have gained increasing attention in the treatment and prognosis prediction of melanoma.<sup>7-9</sup>

Many studies have indicated that TILs are a favorable prognostic factor for melanoma patients, and the presence of TILs might lead to a better prognosis.<sup>10,11</sup> However, some studies did not reveal a significant association between TILs and melanoma prognosis, different TIL responses or subsets have different functions in melanoma.<sup>8,9</sup> Thus, there is currently no conclusive evidence regarding whether TILs are

a robust prognostic factor in melanoma. The conflicting reports on the prognostic value of TILs in melanoma may be due to the heterogeneity of clinical and histologic subtypes, different patient populations, and different TIL grading systems.

Some studies have indicated a distinct correlation between different TIL intensities or grades and different prognosis values; the higher presence of TILs or brisk TILs predict a lower rate of sentinel lymph node (SLN) metastases or favorable survival in melanoma.<sup>11</sup> Considering the great heterogeneity of TILs and their number and distribution within the tumor, most groups have quantified TILs as absent, non-brisk and brisk,<sup>12-16</sup> while others have quantified TILs as absent, mild or scanty, moderate and marked,<sup>11,17,18</sup> moreover, as the TIL grade increases, the hazard ratio of melanoma patient survival is different. However, different subsets of TILs have different or even opposing functions in the tumors.

As a heterogenous group, TILs are implicated in not only effector T cells but also in functionally exhausted T cells, tolerogenic or T regulatory (Treg) cells, dendritic cells (DCs), natural killer (NK) cells, myeloid-derived suppressor cells (MDSCs), macrophages, and other immune cell types.<sup>8,19</sup> As the main antitumor effector cells,<sup>20</sup> CD8+ T lymphocytes comprise the majority of TILs and have been linked to a better prognosis in several types of cancer.<sup>21-23</sup> According to previous reports, CD4+ T lymphocytes exert different functions.<sup>24</sup> Both CD4+ Th1 cells and CD4+ Th2 are related to anti-tumor immunity;<sup>25</sup> however, CD4+ regulatory T cells were observed to inhibit an effective anti-tumor immune

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response.<sup>26</sup> On the other hand, in the adaptive response, the MHC class I and II molecules processed the tumor-associated antigens (TAA) from APCs to the specific receptors of CD8+ and CD4+ T-cells for their activation.<sup>27</sup> Additionally, CD3+ T-cell infiltration into the primary tumor has also been observed as an excellent early predictor of longer survival in metastatic melanoma patients receiving DC-based immunotherapy.<sup>28</sup> Thus, better understanding of the TIL phenotype and function in tumor or the tumor microenvironment is crucial for early prognosis prediction.

Treg cells are the main population involved in maintaining peripheral tolerance.<sup>26</sup> As the most specific Treg marker,<sup>29</sup> FOXP3 expression has been shown to correlate with a poor prognosis in various types of human cancer, including breast cancer and gastrointestinal cancers.<sup>30,31</sup> However, a better prognostic value of Tregs has also been observed in head and neck squamous cell carcinoma.<sup>32,33</sup> For melanoma, most previous studies have suggested that FOXP3+ cells are associated with a favorable prognosis;<sup>34</sup> inconsistently, the controversial results have also been observed by others.<sup>35,36</sup>

In this study, we systematically reviewed the articles for publication about the prognostic roles of TIL responses and CD3+, CD4+, CD8+, FOXP3+, and CD20+ TIL subsets in the prognosis of melanoma. We aimed to include all studies that assessed tumor infiltration with TIL grades, CD3+, CD4+, CD8+ and FOXP3+ lymphocytes between these markers as a prognostic biomarker in melanoma.

## Methods

### Search strategy

A computerized search was performed that included the domain (“melanoma”), the determinant (“Tumor-infiltrating cells”, “TIL”), their synonyms, and a filter for prognostic studies.<sup>37</sup> The publications were gained by searching the PubMed, EMBASE, Sino Med, Springer, Science Direct, The Cochrane Library, and Web of Science databases from the inception of each database to July 6, 2018. There was no restriction on the publication status; however, non-English language studies were excluded. The search strategy, as well as predetermined inclusion and exclusion criteria was based on a previous study,<sup>32</sup> and two researchers independently screened the titles and abstracts based on the criteria. The final selection was made by full-text reading of the selected studies. Discrepancies between the two researchers were resolved by discussion and consensus from another or more researchers. The markers were assessed in two or more studies, and the reference lists of the retrieved articles were also reviewed for sufficient trials.

### Inclusion criteria

According to a previous study,<sup>32</sup> studies were included in which the prognostic value of TILs and CD3+, CD4+, CD8+, FOXP3+, and CD20+ lymphocytes was investigated in patients with melanoma. For TIL response markers, the studies in which TILs were quantified as absent, non-brisk and brisk (focal TIL infiltrate and when TILs present across the entire base or throughout the

substance of the tumor)<sup>14–16</sup> as well as absent, mild or scanty, moderate and marked (focal, multifocal, or diffuse across the entire extent of the tumor)<sup>11,17,18</sup> were included. In this study, both the non-brisk TILs and mild or scanty TILs were defined as non-brisk TILs, the brisk TILs and marked TILs were defined as brisk TILs, but the moderate TILs were analyzed alone. There was no restriction on the detection methods, only the publications concerning lymphocytes in the tumor epithelium were included, and the studies that only investigated lymphocytes in the tumor stroma were excluded. The prognostic value had to be investigated by time-to-event survival analysis with either overall survival (OS), disease-free survival (DFS), relapse/recurrence free survival (RFS), disease-specific survival (DSS) or melanoma-specific survival (MSS). Animal studies, case reports, and commentaries were excluded.

### Data extraction

The data including the author and title, year of publication, biomarker(s), sample size, tumor subsite, tumor stage, scoring methods, cutoffs, and finally outcome of univariate and multivariate analysis defined by the hazard ratio (HR), 95% confidence interval (CI), and p-values were obtained from each included publication. When these parameters were not mentioned in the article but the Kaplan-Meier curves were available, the data from the Kaplan-Meier curves were extracted and digitized using the open-source Engauge Digitizer software (<http://digitizer.sourceforge.net/>), and the univariate HR was estimated.<sup>38,39</sup> When HRs were not mentioned, Kaplan-Meier curves were not available, or HRs did not match the shown Kaplan-Meier curves, the studies were excluded from the meta-analysis.

### Assessment of study quality

This study is compliant with the PRISMA checklist.<sup>40</sup> All the relevant publications were appraised for the risk of bias using the Quality and Prognosis Studies (QUIPS) criteria, a validated and useful tool for systematic reviewers for the critical appraisal of study quality.<sup>41</sup> According to the previous study,<sup>32</sup> the risk of bias was scored as low, moderate or high for six different domains: study participation, study attrition, prognostic factor measurement, outcome measurement, study confounding and statistical analysis, and reporting. For this systematic review, there was no restriction on the treatment modality, and studies that mentioned treatment methods were rare. The risk of bias was assessed by two researchers independently. Differences were resolved by discussion.

### Statistical analysis

According to a previous study,<sup>32</sup> HRs were used that described the risk of events for high TILs versus low TILs. If the study reported the HR for low TILs vs. high TILs, the reciprocal was taken. The meta-analysis and creation of the forest plots was performed in Stata11.0 software.

## Results

### Study selection and basic characteristics

The PubMed, EMBASE, Sino Med, Springer, Science Direct, The Cochrane Library, and Web of Science databases search gained 5,492 hits and yielded 3,303 hits after removing duplicates (Figure 1). Of the 298 publications that remained after the screening of titles and abstract, 6 were non-English, 47 were reviews or commentaries, 4 were unavailable, and 169 were mismatched with our domain, determinant and outcomes. Therefore, 72 full-text articles were assessed, among which 41 met our inclusion criteria and were included in the final analysis.<sup>4,10-13,15-18,28,34-36,42-70</sup> Of the excluded literature, 11 studies reported no hazard ratios or Kaplan-Meier curves; 9 studies reported the outcome only in terms of metastasis survival or metastasis-free survival; 6 studies reported the prognostic role of TILs in multiple tumors, including melanoma, and the hazard ratios or Kaplan-Meier curves in melanoma were not available; 5 studies evaluated FOXP3 expression in tumor cells instead of TILs or TILs in tumor stroma. The study characteristics of the remaining 41 studies that were included in the final meta-analysis are shown in Table 1. All the enrolled studies in our study detected TILs at the same time of pathological diagnosis or used the tissue samples of pathological diagnosis to detect TILs. Almost all studies investigated TILs by immunohistochemistry or hematoxylin- and eosin (H&E)-stained sections in paraffin-embedded tissue except the study by Knol et al.

Furthermore, we also paid attention to the definition of time-to-event variables. In a few studies which mentioned the specific treatment,<sup>28,43,58,59</sup> OS was defined as the time from the date of start of the treatment to death or last follow-up, DFS was defined as the time from the date of start of the treatment to the date of first recurrence and/or disease progression (regional or distant metastases) or last follow-up, RFS was defined as the time from the date of start of the treatment to the date of the clinical recurrence. In two studies involved in Sentinel lymph node biopsy (SLNB),<sup>16,52</sup> RFS and OS were calculated from SLNB to an event (either recurrence or death) or until last follow-up. In the remaining studies, OS was defined as the time from pathological diagnosis to the time of death or last follow-up, DFS was defined as the time from pathological diagnosis to the date of first recurrence and/or disease progression (regional or distant metastases) or last follow-up, RFS was defined as the time from pathological diagnosis to the date of the recurrence. DSS or MSS was calculated from the date of pathological diagnosis to the date of death from melanoma or last follow-up.

### Summary of the quality and risk of bias of the included studies

According to the QUIPS criteria system, the risk of bias of the 41 studies remaining after full-text screening was critically evaluated, and all the literature was evaluated by 2 independent reviewers; if a difference in evaluation arose, it was solved by

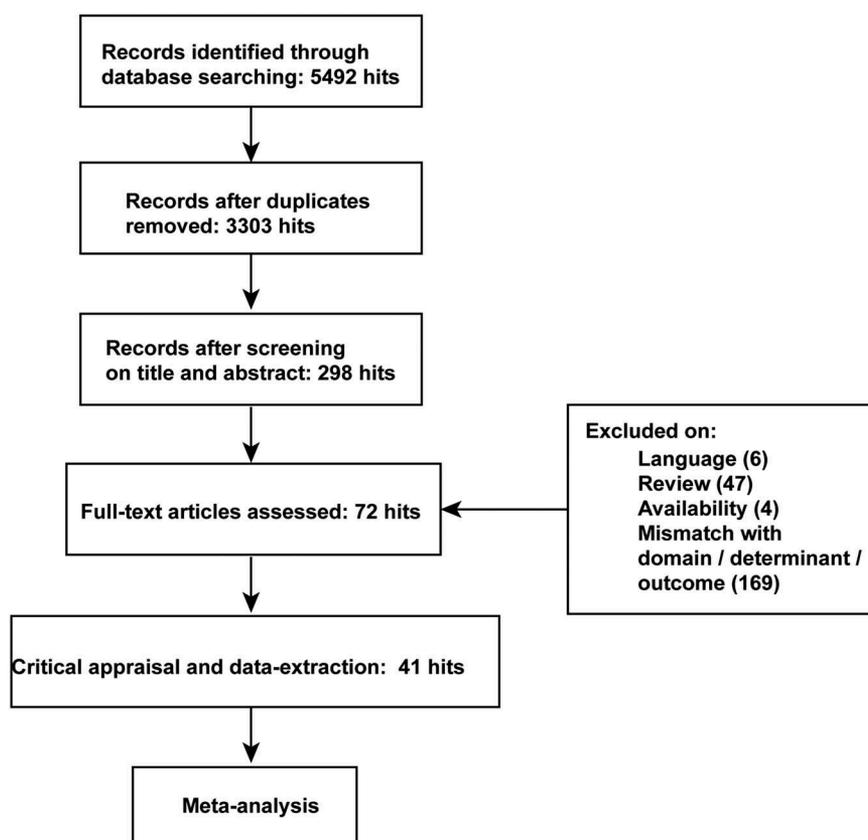


Figure 1. The flow diagram of studies selection and identification.

**Table 1.** Study characteristics of studies included in meta-analysis. RT (Radiotherapy), CT (Chemotherapy), CRT (Chemoradiotherapy), BRAFi (BRAF inhibitor), IM (Immunotherapy), DC-V (DC vaccinations), BCT (Biochemotherapy), OS (Overall Survival), DFS (Disease-free survival), RFS (Relapse/Recurrence free survival), DSS (Disease-specific survival), MFS (Metastasis-free survival), MSS (Melanoma-specific survival), PFS (Progression-free survival), CSS(Cancer-specific survival), BMS (Brain metastases survival), CM (cutaneous melanoma), ALM (acral lentiginous melanoma), UM (uveal melanoma), AM (acral melanoma), Melanoma (M) was defined as the type of melanoma that is not explicitly stated in the studies.

Study	Sample size	Stage	Breslow thickness	Clark level	Treatment	Biomarkers	Survival	Material	Technique	Melanoma
Sinimon AJ 2018 <sup>16</sup>	1367	?	≥0.70mm	?	?	TILs (nonbrisk, brisk)	OS	FFPE	H&E	CM
Letcha AF 2018 <sup>11</sup>	173	?	all	?	?	TILs (Mild, Moderate, Marked)	OS, DFS	FFPE	H&E	CM
Balaton T 2017 <sup>43</sup>	30	III/IV	?	?	CT/CRT/BRAFi+IM	CD4, CD8, FoxP3, CD16, CD68	OS	FFPE	IHC	CM
Lee WJ 2018 <sup>13</sup>	122	all	all	?	?	TILs, PD1, PD-1 <sup>+</sup> TILs	OS	FFPE	IHC	CM
Melsted WN 2017 <sup>61</sup>	200	?	all	?	?	FoxP3	OS, DFS, MSS	FFPE	IHC	CM
Castaneda CA 2017 <sup>44</sup>	537	all	all	I-V	?	TILs (Mild, Moderate, Marked)	OS	FFPE	H&E	ALM
Tas F 2017 <sup>65</sup>	750	all	all	I-V	?	TILs (Mild, Moderate, Marked)	OS, RFS	FFPE	H&E	CM
Weiss SA 2016 <sup>70</sup>	1241	?	all	?	?	TILs (nonbrisk, brisk), CD3, CD45, FoxP3	OS, RFS	FFPE	IHC/H&E	CM
Massi D 2017 <sup>59</sup>	64	M1	?	?	BRAFi	CD8, CD103, FoxP3	OS, PFS	FFPE	IHC	M
Saldanha G 2017 <sup>64</sup>	655	I/II	?	?	?	TILs (nonbrisk, brisk)	OS, DSS, MFS	FFPE	H&E	CM
Melief SM 2017 <sup>60</sup>	73	IV	?	?	?	CD4, CD8, FoxP3	OS	FFPE	IHC/IF	M
Lagouros E 2009 <sup>35</sup>	44	?	?	?	?	CD3, CD4, CD25, FoxP3	OS	FFPE	IHC	UM
Vasaturo A 2016 <sup>58</sup>	77	III/M1	all	?	DC-V, CT	CD3	OS	FFPE	IHC	CM
Kakavand H 2015 <sup>52</sup>	60	III/IV	?	III-V	?	CD3, CD4, CD8, FoxP3	OS, RFS	FFPE	IHC	M
de Moll EH 2015 <sup>10</sup>	94	I-III	?	?	?	TILs (nonbrisk, brisk), CD2	OS, RFS	FFPE	IHC/IF	CM
Massi D 2015 <sup>58</sup>	80	M1	?	?	BRAFi	TILs (present)	OS, PFS, MSS	FFPE	IHC/H&E	M
Fortes C 2015 <sup>18</sup>	4133	?	all	?	?	TILs (scanty, moderate, marked)	OS	FFPE	H&E	CM
Donizy P 2015 <sup>46</sup>	104	all	all	I-V	?	TILs (nonbrisk, brisk)	OS, CSS, DFS	FFPE	IHC/H&E	CM
Madore J 2015 <sup>56</sup>	51	IV	?	?	?	TILs (mild, moderate, marked)	MSS	FFPE	IHC/H&E	CM
Thomas NE 2013 <sup>67</sup>	2845	all	all	?	?	TILs (nonbrisk, brisk)	OS, MSS	FFPE	H&E	CM
Cintolo JA 2013 <sup>45</sup>	161	T4	≥4mm	?	?	TILs (present)	MSS	FFPE	IHC/H&E	M
Lee SJ 2013 <sup>35</sup>	90	?	all	?	?	TILs (nonbrisk)	OS	FFPE	IHC/H&E	AM
Grotz TE 2013 <sup>48</sup>	250	all	all	II-V	?	TILs (nonbrisk, brisk)	RFS, DFS, MSS	FFPE	H&E	CM
Knol AC 2012 <sup>33</sup>	47	III	?	?	TIL	TILs (present)	OS, RFS	Fozen	FACS/IHC	M
Azimi F 2012 <sup>42</sup>	1865	all	≥0.75mm	II-V	?	TILs grade	RFS, MSS	FFPE	H&E	M
Holtan SG 2012 <sup>51</sup>	118	III/IV	?	?	?	CD20	OS	FFPE	IHC	M
Erdag G 2012 <sup>47</sup>	147	III/IV	?	?	?	CD45, CD3, CD8, CD20, CD138	OS	FFPE	IHC	M
Knol AC 2011 <sup>34</sup>	102	III	all	?	?	FoxP3	OS, PFS	Fresh	qRT-PCR	M
Burton AL 2011 <sup>12</sup>	515	all	≥1mm	II-V	?	TILs (nonbrisk, brisk)	OS, DFS	FFPE	H&E	CM
Rao UN 2010 <sup>63</sup>	293	T4	all	?	?	TILs (nonbrisk, brisk)	OS, RFS	FFPE	H&E	CM
Mougiakakos D 2010 <sup>36</sup>	100	?	?	?	?	FoxP3, CD4	OS	FFPE	IHC	UM
Bogunovic D 2009 <sup>17</sup>	38	III/IV	?	?	RT, IM, CT, ?	CD3, TILs grade	OS	FFPE	IHC/H&E	M
Mandala M 2009 <sup>57</sup>	1251	I-II	all	I-V	?	TILs (nonbrisk, brisk)	OS, DFS	FFPE	H&E	CM
Tuomaala S 2007 <sup>68</sup>	85	?	?	?	?	TILs (few, moderate, many)	OS	FFPE	H&E	CM
Hillen F 2008 <sup>50</sup>	58	?	all	?	?	CD3, CD16, CD20, CD69, FoxP3	OS	FFPE	IHC	CM
Taylor RC 2007 <sup>66</sup>	887	all	all	I-V	?	TILs (nonbrisk, brisk)	DFS	FFPE	H&E	CM
Piras F 2005 <sup>62</sup>	47	I/II	?	II-V	?	CD8	OS	FFPE	IHC	CM
Ladányi A 2004 <sup>54</sup>	76	?	all	?	?	CD25, OX40	OS	FFPE	IHC	CM
Häkansson A 2001 <sup>49</sup>	41	?	all	?	BCT	CD4	OS, PFS	FFPE	IHC	M
Tuthill RJ 2002 <sup>69</sup>	259	?	all	?	?	TILs (nonbrisk, brisk)	OS, PFS	FFPE	H&E	CM
Clemente CG 1996 <sup>15</sup>	285	?	all	?	?	TILs (nonbrisk, brisk)	OS	FFPE	H&E	CM
Kluger HM 2015 <sup>4</sup>	95	IV	?	?	IM, ?	CD3, CD4, CD8, FoxP3	OS, BMS	FFPE	IF	M

discussion. Consistent with previous studies in head and neck squamous carcinoma,<sup>32</sup> very few articles mentioned information about patients who were lost to follow up or study attrition, and most studies did not use consecutive cohorts. Because no consensus exists on cut-offs, several studies were unclear about their scoring methods and data-dependent cut-offs. The complete quality assessment of the publications included in the meta-analysis is shown in Supplement Table 1.

### TILs as prognostic biomarkers and the correlation between different TIL grades and prognosis value

Many studies have reported that TILs are a prognostic factor for melanoma patients and have indicated a distinct correlation between different TIL intensities or grades and prognosis value. Thus, the prognostic value of TILs present was assessed in 8 studies that were eligible for inclusion in the meta-analysis, among which 1 study analyzed the prognostic roles of TILs in two different subtypes of melanoma (nail unit melanoma and non-nail unit acral melanoma). As shown in Figure 2, although no significant difference was found, the pooled meta-analysis showed an advantage trend for TILs present [pooled HR: 0.65 (0.42–1.00)] for OS. Additionally, we assessed the prognostic roles of different TIL grades for the OS of melanoma patients, and the results indicated that an advantageous prognostic role for non-brisk TILs [HR: 0.71 (0.55–0.90)] and brisk TILs [HR: 0.61 (0.52–0.72)] but not moderate TILs [HR: 0.81 (0.32–2.07)] for OS (Figure 3).

For the prognostic roles of TILs for DFS, no study mentioned the prognostic roles of TILs present for DFS; thus, we directly assessed the correlation between different TIL grades and DFS. The results of the meta-analysis showed no obvious predictive roles of TILs for DFS, among which non-brisk TILs were assessed in 2 studies [HR: 0.82 (0.59–1.12)], moderate TILs were assessed in only 1 study [HR: 0.88 (0.32–2.41)], and

brisk TILs were assessed in 5 studies [HR: 0.74 (0.34–1.62)] (Figure 4).

For the prognostic roles of TILs for RFS, 3 studies were eligible for inclusion in the meta-analysis of TILs present, and the results showed an advantageous prognostic value [HR: 0.72 (0.58–0.90)] (Figure 5(a)). No study mentioned the prognostic value of moderate TILs for RFS, and only one study involved the prognostic role of non-brisk TILs for RFS [HR: 0.57 (0.32–1.01)] (Figure 5(b)). The prognostic value of brisk TILs was assessed in 3 studies, and the meta-analysis demonstrated brisk TILs to be a favorable prognostic factor for RFS [HR: 0.50 (0.27–0.94)] (Figure 5(b)).

The prognostic value of TILs present for DSS/MSS was analyzed in 4 studies by meta-analysis, and the results showed an obviously favorable prognostic role [HR: 0.46 (0.30–0.70)] (Figure 6(a)). Furthermore, the 2 studies that reported non-brisk TILs also yielded a conclusive result [HR: 0.69 (0.49–0.97)], the 4 studies that involved brisk TILs showed a better prognostic role [HR: 0.31 (0.19–0.52)] (Figure 6(b)).

Subsequently, the prognosis value of different TIL grades in different types of melanoma was analyzed. For OS, TILs present showed a favorable prognostic role in unspecified melanoma patients (that was defined as the type of melanoma that is not explicitly stated in the studies) [HR: 0.43 (0.27–0.67)], but not in cutaneous melanoma ( $P=0.475$ ) (Table 2, Supplement Figure 1). Interestingly, brisk TILs showed an advantageous prognostic value in cutaneous melanoma from 12 studies [HR: 0.61 (0.52–0.72)] and only one study involved the OS in unspecified melanoma (Table 2, Supplement Figure 3). Consistent with the pooled analysis, moderate TILs did not show a favorable prognostic role in cutaneous melanoma and unspecified melanoma (Table 2, Supplement Figure 2). For DSS/MSS, only TILs present were involved in 4 studies and showed a good prognostic value in unspecified melanoma but not cutaneous melanoma (Table 2, Supplement Figure 4). For DFS and RFS, all the studies included in this meta-analysis were performed in cutaneous melanoma.

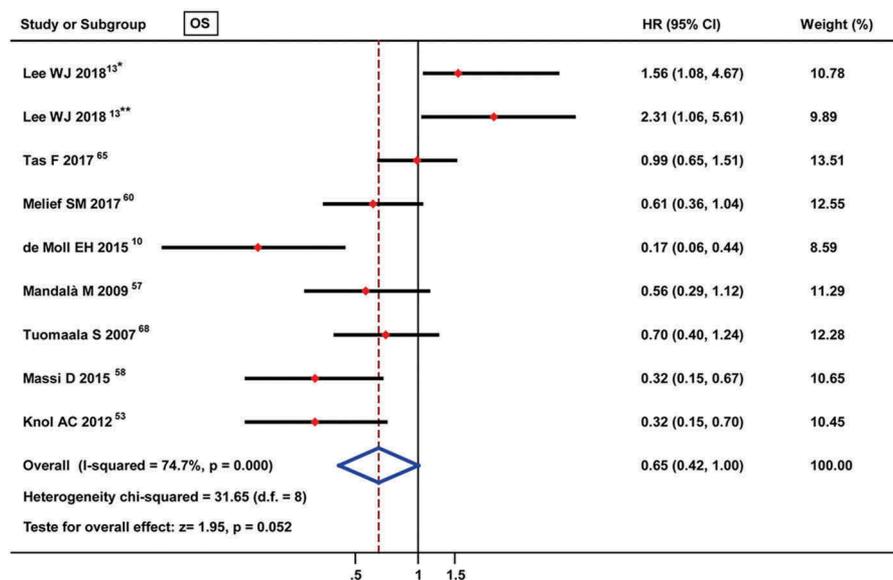


Figure 2. Forest plots of prognostic value of TILs present on overall survival in melanoma patients. \* nail unit melanoma, \*\* non-nail unit acral melanoma.

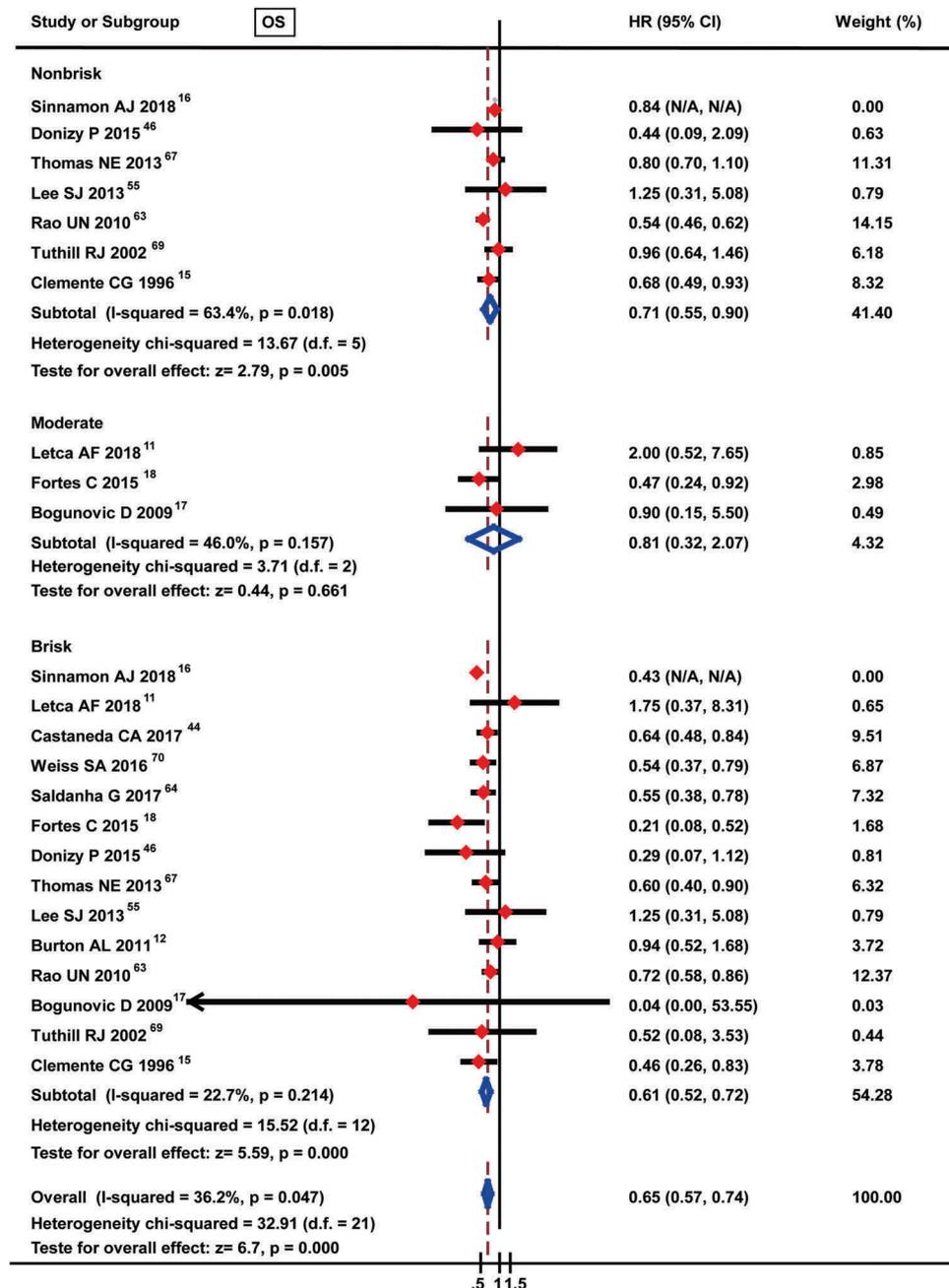


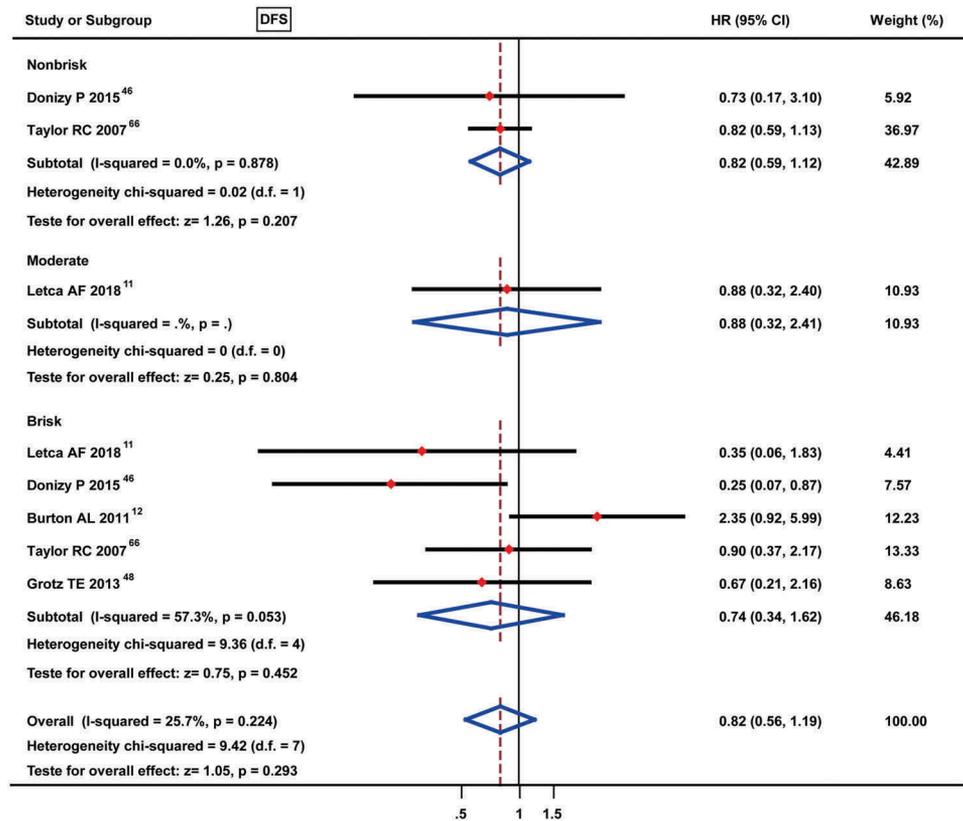
Figure 3. Forest plots of prognostic value of different TIL grades on overall survival in melanoma patients.

### CD3+ TILs as a prognostic biomarker

The prognostic value of CD3+ TILs was assessed in 8 studies, among which 7 were included in the analysis and 1 was excluded from this meta-analysis because of the lack of a survival curve and 95% confidence interval (CI). The results showed an advantage for high CD3 TIL infiltration [HR: 0.57 (0.41–0.79)] for OS. Only one study that reported RFS also yielded a similar conclusive result [HR: 0.36 (0.17–0.76)] (Table 3, Supplement Figure 5). However, no study analyzed the DFS and DSS/MSS. For the subgroup analysis, CD3+ TILs indicated a favorable prognostic role for OS in unspecified melanoma patients in 4 studies [HR: 0.55 (0.4–0.77)], but not in cutaneous melanoma and uveal melanoma (Table 4, Supplement Figure 10).

### CD4+ TILs as a prognostic biomarker

Similar to the studies of CD3+ TILs, 7 studies were assessed for CD4+ TILs: 6 articles were finally included in the meta-analysis, and 1 was excluded because of the lacking of a survival curve and CI values. As shown in Figure 8, high CD4+ TILs are a favorable prognostic factor for the OS of melanoma patients according to the HR [0.56 (0.37–0.85)]. For RFS, only one study was reported and showed a similar favorable prognostic role of the HR [0.34 (0.15–0.77)] (Table 3, Supplement Figure 6). No study has reported on the DFS and DSS/MSS. For the subgroup analysis, CD4+ TILs showed an advantage prognostic role for OS in cutaneous melanoma in only one study [HR: 0.22 (0.09–0.55)] and in unspecified



**Figure 4.** Forest plots of prognostic value of different TIL grades on disease-free survival in melanoma patients. No data were available for the TILs present and only 1 study was met the moderate TILs.

melanoma in 4 studies [HR: 0.6 (0.37–0.97)], but not in uveal melanoma with only one study (Table 4, Supplement Figure 11).

### CD8+ TILs as a prognostic biomarker

For the prognostic value of CD8+ TILs, we searched 7 studies that met the enrollment requirements for OS and analyzed by meta-analyses. The results showed a favorable prognostic value for CD8+ TIL infiltration [HR: 0.50 (0.37–0.69)] (Figure 9). Only one study reported the RFS and indicated an advantageous prognostic value [HR: 0.42 (0.21–0.84)] (Table 3, Supplement Figure 7). Consistent with the studies on CD3+ and CD4+ TILs, no study has reported on the DFS and DSS/MSS of CD8+ TILs in melanoma. For the subgroup analysis, CD8+ TILs showed a good prognostic value for OS in cutaneous melanoma in 2 studies [HR: 0.38 (0.19–0.75)] and unspecified melanoma in 5 studies [HR: 0.53 (0.37–0.75)], but no study has reported in uveal melanoma (Table 4, Supplement Figure 12).

### FOXP3+ TILs as a prognostic biomarker

Ten studies reported results on the prognostic value of FOXP3+ TILs, among which 1 lacked survival curve and 95% CI values, and the remaining 9 studies were included in the meta-analysis. The results indicated a better OS in melanoma patients with high FOXP3 TIL infiltration [HR: 0.57 (0.40–0.82)] (Table 3, Supplement Figure 8). A comparable trend was observed for RFS

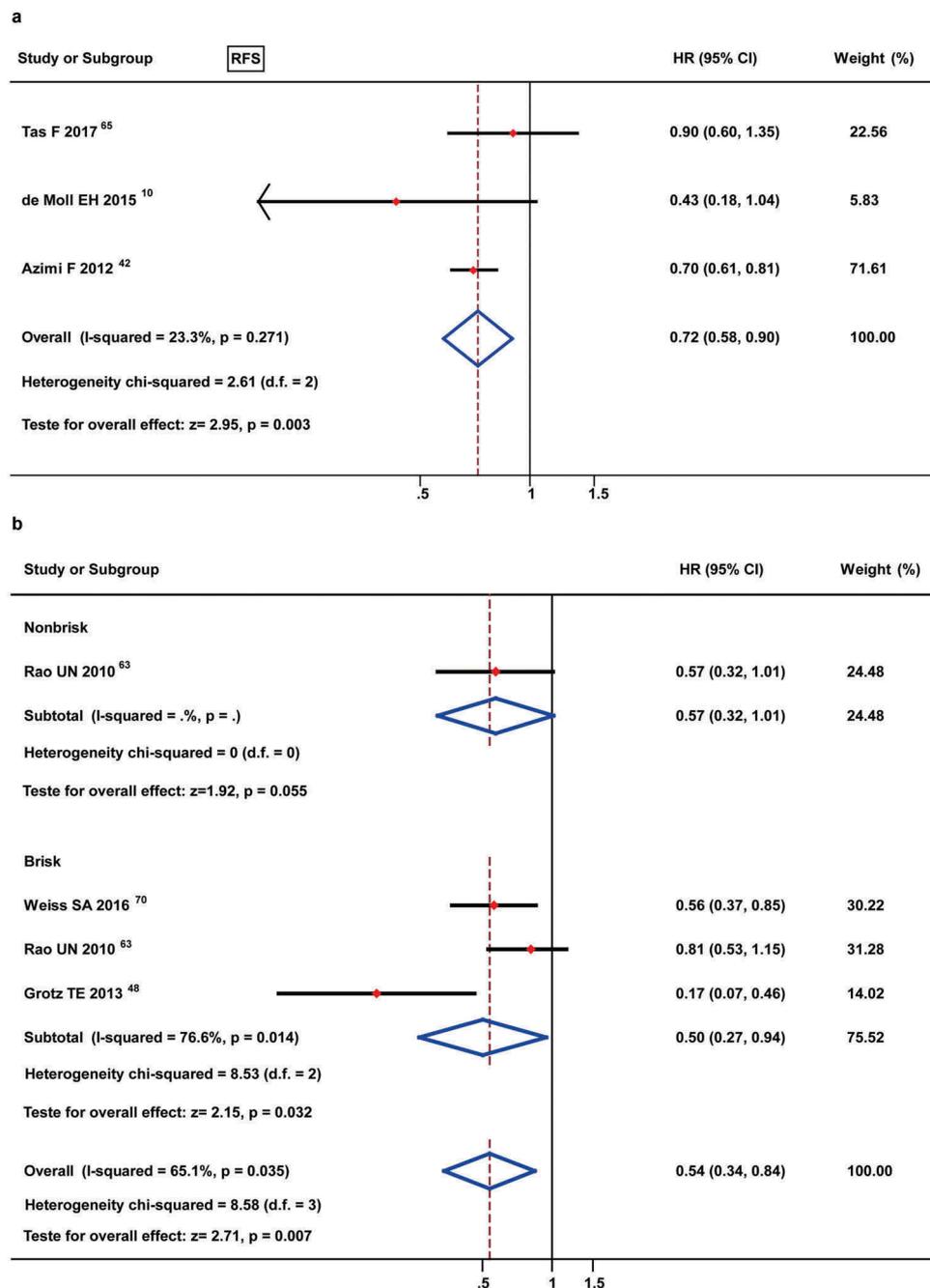
with only one study [HR: 0.60 (0.31–1.17)]. No study reported on DFS and DSS/MSS. For the subgroup analysis, FOXP3+ TILs showed a favorable prognostic role for OS in cutaneous melanoma patients in 4 studies [HR: 0.58 (0.36–0.95)] and unspecified melanoma in 4 studies [HR: 0.45 (0.28–0.73)], but not in uveal melanoma with only one study (Table 4, Supplement Figure 13).

### CD20+ TILs as a prognostic biomarker

For the prognostic value of CD20+ TILs, we searched 3 studies that met the enrollment requirements for OS. The meta-analysis showed a markedly favorable prognostic value for patients with high CD20 TIL infiltration [HR: 0.49 (0.34–0.71)] (Table 3, Supplement Figure 9). However, no study has reported on the DFS, RFS and DSS/MSS of CD20+ TILs in melanoma. For the subgroup analysis, CD20+ TILs indicated a good prognostic value for OS in unspecified melanoma in 2 studies [HR: 0.49 (0.34–0.72)], but no in cutaneous melanoma with only one study, and no study has reported in uveal melanoma (Table 4, Supplement Figure 14).

## Discussion

Melanoma is the most aggressive skin cancer. Thus, accurate prognostic prediction is paramount for the selection of appropriate therapies and management of melanoma patients; however, robust biomarkers are currently lacking. Considering that melanoma is considered an “immunogenic” tumor, the

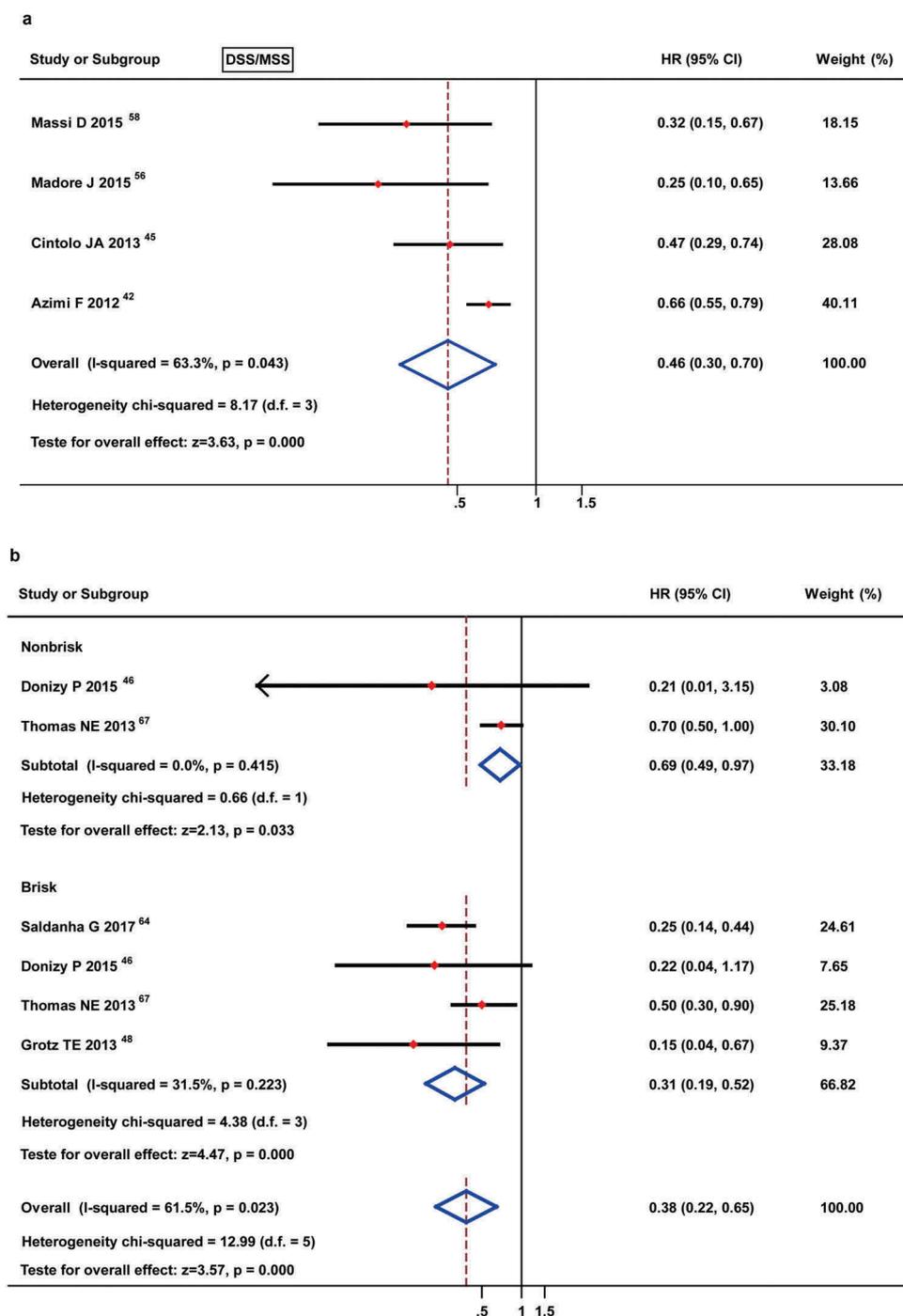


**Figure 5.** Forest plots of prognostic value of TILs present (A) and different TIL grades (B) on progression-free survival in melanoma patients. Only 1 study was met the non-brisk TILs and no data were available for the moderate TILs.

principal goal of our study was to investigate the prognostic role of the TIL response and different grades and subsets of TILs to predict the outcome of melanoma.

First, we investigated the prognostic efficacy of the TIL response by meta-analysis to provide a quantitative synthesis of several studies. Our analysis indicated that TILs present correlated with a favorable prognosis for the OS, RFS and DSS/MSS of melanoma. This finding was consistent with a hypothesis proposed in 1978 that concluded from a retrospective study including 669 melanoma patients that a dense lymphocytic infiltrate in primary tumors improved their survival rates.<sup>71</sup> This is also supported by the findings in subsequent studies of melanoma<sup>10</sup> and fit with studies in other tumors.<sup>32</sup>

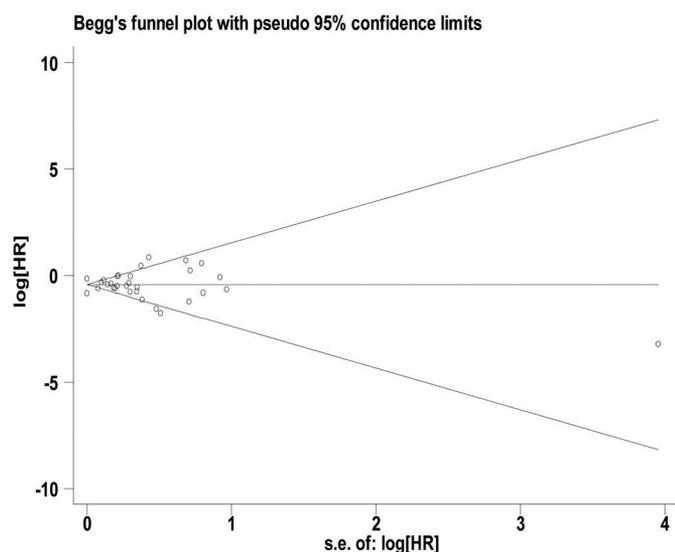
A distinct correlation is found between different TIL intensities or grades and different prognosis values in melanoma. Thus, the establishment of a graded system to categorize the extent of TIL involvement within a tumor has become the first requirement to assess of the role of TILs in prognostication. Currently, two main comprehensive classification systems are commonly used in melanoma, and most groups quantify TILs as absent, non-brisk and brisk (focal TIL infiltrate and when TILs present across the entire base or throughout the substance of the tumor),<sup>14-16</sup> while others quantify TILs as absent, mild or scanty, moderate and marked (focal, multifocal, or diffuse across the entire extent of the tumor).<sup>17,18</sup> In this study, both the non-brisk TILs and mild or scanty TILs



**Figure 6.** Forest plots of prognostic value of TILs present (**A**) and different TIL grades (**B**) on disease-specific survival or melanoma-specific survival in melanoma patients. No data were available for the moderate TILs.

were defined as non-brisk TILs, the brisk TILs and marked TILs were defined as brisk TILs, and the moderate TILs were analyzed alone. The meta-analysis demonstrated a favorable prognostic role of non-brisk TILs [HR: 0.71 (0.55–0.90) for OS, HR: 0.57 (0.32–1.01) for RFS, and HR 0.69 (0.49–0.97) for DSS/MSS]. Noteworthy, a better favorable prognostic value of TILs was observed in the brisk TIL grade with HR 0.61 (0.52–0.72) for OS, HR 0.50 (0.27–0.94) for RFS, and HR 0.31 (0.19–0.52) for DSS/MSS. However, TILs of any grade showed no obviously predictive effect on DFS. Thus, our study draws a conclusion that TILs are a favorable prognostic

factor in melanoma, and the larger is the infiltration area of TILs, the better is the prognosis of melanoma patients. This finding is consistent with the reports of Clemente et al and Clark et al,<sup>14,15</sup> both of whom observed that the survival rate of melanoma patients with a brisk TIL response compared with those with a non-brisk response was higher, and the TIL response was an independent prognostic indicator. The possible reason for the favorable role of TILs in the survival of melanoma patients is that, TILs is not just inflammatory cells but rather antitumor effector cells that are recruited to the tumor as a part of the specific immune response. In addition,



**Figure 7.** Funnel plot of of TILs present on overall survival.

the presence of TILs would decrease the involvement of regional lymph nodes and distant metastasis to lead a positive outcome.<sup>65</sup>

Considering the great heterogeneity of the TIL phenotype, the phenotype of TIL cells from different tumor sources varies greatly. Histologically, most TILs express CD3 and CD3 as a general TIL marker.<sup>72,73</sup> Although some studies have reported that the CD3-TCR complex should be helpful in the regulation of the signal transduction and CD3 is usually used as a pan-T cell marker,<sup>74–76</sup> the exact function of CD3 TILs in the prognosis of melanoma patients is currently unclear. In this study, the prognostic value of CD3+ TILs was assessed in 7 studies and found that CD3 TIL infiltration is a favorable prognostic factor for overall survival in melanoma. This finding is consistent with the results of meta-analysis in squamous cell carcinoma of the head and neck.<sup>32</sup> The specific mechanism of CD3+ TILs as favorable prognostic factor is unclear, and may be related to its regulation on cell signal transduction.

T cells present in tumors are mixtures of CD8+ and CD4+ cells, and CD8+ TILs and CD4+ TILs are the main cell populations of the TIL treatment system, in which CD8+ TILs kill tumor cells and CD4+ TILs show immunomodulatory effects.<sup>77,78</sup> The proportion of CD4+ and CD8+ TILs in different tumor tissues was different, reflecting the changes in the body's immune status.<sup>79</sup> The prognostic value of CD8+ TILs was most frequently assessed in various tumors. For the prognostic role of CD8+ TILs in melanoma, we assessed 7 studies by meta-analysis and showed a better favorable prognostic value for the OS of melanoma patients compared with CD3+ TIL infiltration. This may be closely related to the directly killing effect of CD8+ TILs on tumors cells.

Recently, the role of CD4+ T cells in tumor immunity has received extensive attention; CD4+ T cells play an important role in the initiation of cytotoxic T lymphocytes (CTLs) as well as in the formation and functional maintenance of memory CD8+ T cells. In tumors, after CD4+ T cells are activated, CTLs can be activated by activated CD4+ T cells through various mechanisms to maintain and enhance CTL's anti-tumor response.<sup>80,81</sup> Except for the immunomodulatory effects, CD4+ TILs function as effector cells to aid the killing of tumor cells by CD8+ TILs and even independently clear tumor cells that are resistant to CD8+ cells.<sup>81–83</sup> Currently, the prognostic role of CD4+ TILs has gain much attention. Our study enrolled 6 studies into the meta-analysis and showed that CD4+ TILs are a favorable prognostic factor for the OS of melanoma patients. Thus, the favorable role of CD4+ TILs in the survival of melanoma patients is probably closely related to above-mentioned functions of CD4+ TILs.

Generally, the infiltration of Treg cells into tumors inhibits antitumor immune responses, and the depletion of Treg cells enhance anticancer treatments.<sup>84</sup> Thus, understanding the markers of Treg cells is essential for the studies of Treg cells in tumors. It has been confirmed that CD25 and transcription factor FOXP3 are highly expressed in Treg cells and FOXP3 is the most specific marker.<sup>29</sup> Several studies have found a close association between FOXP3 expression and the prognosis of patients in various tumors.<sup>30,31</sup> However, the controversial

**Table 2.** Summary of the prognostic value of different TIL status on OS and DSS/MSS in different types of melanoma. CM (cutaneous melanoma), UM (uveal melanoma), Melanoma (M) was defined as the type of melanoma that is not explicitly stated in the studies.

TIL status	Subgroup	OS				DSS/MSS			
		No of studies	$I^2$ (P)	HR (95% CI)	P	No of studies	$I^2$ (P)	HR (95% CI)	P
Present	CM	6	75.7% (0.001)	0.82 (0.47–1.42)	0.475	2	74.9% (0.046)	0.45 (0.18–1.15)	0.095
	M	3	27.5% (0.252)	0.43 (0.27–0.67)	<0.001				
Moderate	CM	2	72% (0.059)	0.86 (0.21–3.48)	0.830	2	0% (0.393)	0.42 (0.28–0.63)	<0.001
	M	1		0.9 (0.15–5.45)	0.909				
Brisk	CM	12	26.8% (0.181)	0.61 (0.52–0.72)	<0.001	1	0.6 (0.31–1.17)	0.015	0.015
	M	1		0.04 (0–92.56)	0.415				

**Table 3.** Summary of the prognostic value of different TIL phenotypes on overall survival (OS) and relapse/recurrence free survival (RFS) in melanoma patients.

Subgroup	OS				RFS			
	No of studies	$I^2$ (P)	HR (95% CI)	P	No of studies	$I^2$ (P)	HR (95% CI)	P
CD3+ TILs	7	27.9% (0.215)	0.57 (0.41–0.79)	0.001	1	0.36 (0.17–0.76)	0.007	
CD4+ TILs	6	62.6% (0.02)	0.56 (0.37–0.85)	0.006	1	0.34 (0.15–0.77)	0.01	
CD8+ TILs	7	44.3% (0.096)	0.5 (0.37–0.69)	<0.001	1	0.42 (0.21–0.84)	0.015	
FOXP3+ TILs	9	46.4% (0.061)	0.57 (0.4–0.82)	<0.001	1	0.6 (0.31–1.17)	0.015	
CD20+ TILs	3	0% (0.748)	0.49 (0.34–0.71)	<0.001				

**Table 4.** Summary of the prognostic value of different TIL phenotypes on OS in different types of melanoma. CM (cutaneous melanoma), UM (uveal melanoma), Melanoma (M) was defined as the type of melanoma that is not explicitly stated in the studies.

TIL phenotypes	Subgroup	No of studies	$I^2$ (P)	HR (95% CI)	P
CD3+ TILs	CM	3	51% (0.130)	0.93 (0.27–3.15)	0.906
	UM	1		5.5 (N/A- N/A)	0.03
	M	4		0.55 (0.4–0.77)	<0.001
CD4+ TILs	CM	1	28.4% (0.241)	0.22 (0.09–0.55)	0.001
	UM	1		0.80 (0.50–1.27)	0.346
	M	4		0.60 (0.37–0.97)	0.039
CD8+ TILs	CM	2	55.6% (0.080)	0.38 (0.19–0.75)	0.005
	M	5		0.53 (0.37–0.75)	<0.001
	M	5		50.4% (0.089)	0.53 (0.37–0.75)
FOXP3+ TILs	CM	4	0% (0.785)	0.58 (0.36–0.95)	0.032
	UM	1		1.14 (0.68–1.91)	0.617
	M	4		0.45 (0.28–0.73)	0.001
CD20+ TILs	CM	1	47.6% (0.126)	0.42 (0.06–2.91)	0.380
	M	2		0.49 (0.34–0.72)	<0.001
	M	2		0% (0.456)	0.49 (0.34–0.72)

results have always existed in melanoma. Most previous studies have suggested that FOXP3+ cells are associated with a favorable prognosis,<sup>34,43</sup> while others have shown that FOXP3+ cells are a poor prognosis factor.<sup>35,36</sup> Therefore, 9 studies were included in our meta-analysis for the prognostic value of FOXP3+ TILs and demonstrated that FOXP3 TIL infiltration is a beneficial prognosis factor in melanoma. This is inconsistent with the standpoint that the infiltration of Treg cells inhibits the antitumor immune response.

The first possible explanation for this inconsistency is that, Tregs suppress the inflammatory response and disturb metabolism which are associated with tumor progression by secreting anti-inflammatory cytokines and growth factor such as IL-10, IL-35 and tumor growth factor-(TGF-)  $\beta$ .<sup>84</sup> Another possible reason is that the FoxP3+ TILs could be just a basic reflection of Tregs, the ratio of FOXP3+ Tregs to other lymphocyte subsets and CD8+/FOXP3+ T cell ratio are the potential effective indicator of the quantity of Tregs.<sup>32,33</sup> However, almost no study assessed these ratios in melanoma, only one study assessed the prognostic role of CD8/Tregs ratio (Tregs were defined as CD3+ CD8-FOXP3+) HR 1.637 (0.956–2.801) for OS.<sup>60</sup> The last possible explanation for the favorable role of FOXP3+ TILs is that, FOXP3 is not specific for activated Tregs, it is needed to conjoint assess FOXP3 and additional markers, such as CD25. Regretfully, no study assessed this combination in melanoma, only two studies assessed the prognostic role of CD25 for OS by HR 1.8 (no 95% CI) ( $P=0.68$ )<sup>35</sup> and two almost identical survival curves ( $P=0.802$ ).<sup>54</sup>

CD20 is a marker of TILs, and the prognostic role of CD20+ TILs was rarely assessed in various tumors. For the prognostic value of CD20+ TILs, we searched 3 studies analyzed by meta-analysis and showed a markedly favorable prognostic value for the patients with high CD20 TIL infiltration in melanoma. The specific mechanism of CD3+ TILs as favorable prognostic factor is unclear, and additional large-sample studies are needed to confirm the results due to the small number of enrolled studies.

Certain limitations of our meta-analysis should be described. First, although the literature search was performed in 7 electronic databases, databases and literature published in other languages except English were not included in our study. Thus, some relevant publications might have been missed.

The main limitation of this study is the heterogeneity within the tumor subgroups included in our meta-analysis.

Although most studies reported in our study were cutaneous melanoma that included many subgroups, very few articles reported the information about patients with uveal melanomas. The prognostic value of biomarkers may be different between different tumor subgroups and tumor stages. Furthermore, most studies did not frequently allow stratification for these different groups. Therefore, the categorizations of patients and tumors could strengthen the conclusions on prognostic biomarkers and provide more insight into the differences between patient or tumor subgroups.

Another limitation of this study is the heterogeneity of the scoring methods and data-dependent cut-offs of biomarkers. Because no consensus exists on cut-offs, several studies were unclear about their scoring methods and data-dependent cut-offs, and different scoring systems and thresholds may lead to different results. Thus, the consensus of the scoring methods and data-dependent cut-offs of TILs would strengthen the conclusions on prognostic biomarkers and provide more insight into the study of TILs.

The heterogeneity in the treatment modality is also a key limitation. Because very few studies mentioned the treatment modality in their reports, our analysis did not consider treatment modalities. However, different treatment modalities have different mechanisms, and the prognostic value of TILs is likely to depend on the given therapy. Therefore, more prognostic studies with different treatment modalities among patient cohorts are needed.

Because the number of retrieved studies was not sufficient to be analyzed depending on the detection methods of biomarkers, no restriction was placed on the detection methods. However, different detection methods may have different TILs, and the prognostic value of TILs is likely to depend on the detection methods. Therefore, additional prognostic studies with H&E staining and immunohistochemistry of TILs are needed in the future.

In conclusion, our meta-analysis confirmed the favorable prognostic role of CD3+, CD4+, CD8+, FOXP3+, and CD20+ TILs in the overall survival of melanoma patients. Brisk TILs are obviously associated with the OS, RFS and DSS/MSS. Thus, TILs showed predictive value for the prognosis of melanoma patients, and detection of TILs status and phenotype in pathological diagnosis would be helpful to guide the treatment and prognosis of patients. However, to incorporate different T-cell markers as predictive biomarkers in clinical

practice, studies using homogeneous patient cohorts according to tumor subsite, stage and treatment are necessary.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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